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Development of an isotope-selective high-performance liquid chromatography detector using chemical-reaction-interface mass spectrometry: application to deuterated cortisol metabolites in urine

Yohannes Teffera and Fred P. Abramson*

The George Washington University Medical Center, Department of Pharmacology, Washington, DC 20037 (USA)

Matt McLean and Marvin Vestal

Vestec, Houston, TX 77054 (USA)

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ABSTRACT

An isotope-selective detector for HPLC based on the particle-beam-interface and the chemical-reaction-interface mass spectrometer (CRIMS) principle is described. This paper focuses on the selective detection of deuterium-labeled analytes. The CRIMS product HD is detected as has been previously described for GC–CRIMS. The analytical performance was not affected by analyte structure or solvent composition. Deuterium detection was linear from 20 ng to 490 ng using $^{2}H_{3}$-labeled cortisol. Based on this method, the fractional abundance of three labeled metabolites of cortisol were determined in the urine of a patient infused with tracer amounts of deuterated cortisol.

INTRODUCTION

Since its introduction in 1982 [1], the chemical-reaction-interface mass spectrometer (CRIMS) in conjunction with GC, has been used in detecting isotopically labeled substances and their metabolites [2–4]. In the CRIMS process, the atoms in the analyte are converted into small polyatomic molecules whose characteristic masses provide selective detection channels when monitored by a conventional mass spectrometer.

When the natural abundance of the isotope of interest is significant (for example ^{15}N and ^{13}C), the observed intensity of the heavy species contains signals derived from both the tracer and the natural abundance of endogenous materials. Selective detection requires subtraction of the endogenous contribution. When the natural abundance of the isotope is lower, as for example for deuterium, the observed intensity, within limits, directly reflects the enriched analyte. Only one GC–CRIMS metabolism study has been conducted utilizing deuterium detection [2,3].

Although HPLC has many advantages over GC, its use with CRIMS has lagged behind GC. Moini and Abramson used a moving belt interface to introduce the HPLC effluents into the chemical reaction interface (CRI) [5]. Recently Abramson *et al.* used the Vestec Universal In-

* Corresponding author.

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terface, a device that efficiently removes HPLC solvents using a helium countercurrent across a membrane, to introduce nonvolatile analytes into the reaction interface [6]. Various ^{15}N and ^{13}C labeled metabolites of caffeine were successfully separated and detected from un-extracted urine with this system.

In this paper we present details of the development of the HPLC–CRIMS device with particular focus on selective detection of ^{2}H-labeled compounds. We also present results for the application of HPLC–CRIMS in the analysis of urinary cortisol metabolites from a patient infused with a tracer level of deuterated cortisol.

EXPERIMENTAL

Chemicals

Acetonitrile and methanol were purchased from EM Sciences, Gibbstown, NJ, USA. Those solvent lots with the lowest levels of evaporation residue (0.1 ppm or less) were used. Trifluoroacetic acid (TFA) was purchased from J.T. Baker, Phillipsburg, NJ, USA. The steroid standards, cortisol (F), cortisone (E), cortisone-21-sulfate, tetrahydrocortisol (THF), 5α-THF, α- and β-cortolone, α- and β-cortol, and tetrahydrocortisone (THE) were purchased from Sigma Chemicals, St. Louis, MO, USA. The labeled standards (9,12,12-$^{2}H_3$)cortisol and (3,5-$^{2}H_2$)tyrosine were purchased from Cambridge Isotopes, Woburn, MA, USA. The gases used in HPLC–MS and HPLC–CRIMS, hydrogen (ultra-high purity grade, 99.995%) and helium (high purity grade, 99.995%), were purchased from Air Products, Allentown, PA, USA.

HPLC–MS and HPLC–CRIMS

A Vestec Universal Interface (Vestec Corporation, Houston, TX, USA) was used to couple the HPLC to a Dupont 21-492 double-focusing mass spectrometer. The schematic for the HPLC–CRIMS setup is shown in Fig. 1. Included are some modifications to the momentum separator component of the Universal Interface as required by the CRI compared to the standard Universal Interface as first described by Vestal *et al.* [7]. For conventional HPLC–MS experiments, the two-stage momentum separator is directly connected to the mass spectrometer using 0.64 cm (1/4″) O.D. tubing. In CRIMS experiments, a single stage momentum separator was connected to the 0.635 cm (± 0.005 cm) (1/4″) O.D., 0.24 cm (3/32″) I.D. alumina tube that passes through the microwave cavity in which the reactions actually take place. This cavity retains the original Bowman configuration [3], but incorporates two modifications. The cavity itself is still cylindrical,

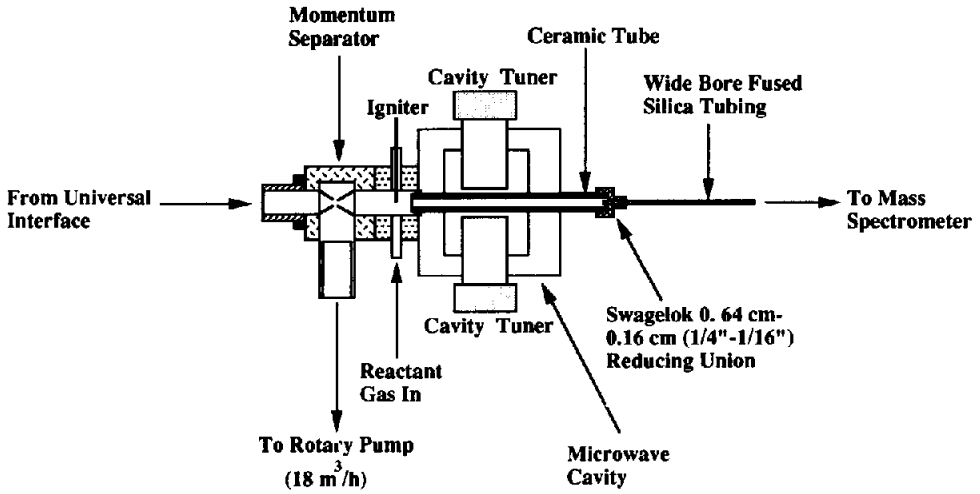

Fig. 1. Schematic for HPLC–CRIMS.

but the external shape has now a rectangular cross-section with thick walls to provide substantially greater resistance to thermal warping. Also, dual cavity tuners are used, as was true for early versions of Bowman's design and as recently reintroduced by Morré and Moini [8]. The ceramic tube is coupled to the MS through a 38 cm length of 0.53 mm I.D. deactivated fused-silica capillary tubing (Hewlett Packard, Avondale, PA, USA). The operation of the Dupont 21-492 mass spectrometer and the data system used for data acquisition has been described elsewhere [3,9]. Electron-impact ionization (EI) was used in all experiments. For HPLC–MS experiments, the mass spectrometer was scanned from 295–375 at a rate of 1.13 s/scan. For CRIMS, hydrogen was the reactant gas and the masses at 26 and 3.022 corresponding to the reaction product C_2H_2 and HD, respectively, were used for single ion monitoring. A resolution of 2000 separated the HD^+ signal from the adjacent H_3^+ signal. The ion source temperature was kept at 200°C for HPLC–MS and at 100°C for CRIMS experiments. The areas of peaks, obtained with a Vector 1 data system (Teknivent, Maryland Heights, MO, USA) with manual setting of the integration limits, were taken as the MS response.

HPLC separation

HPLC analyses were performed using a reversed-phase system on a 150 mm × 4.6 mm I.D., 5μm C_{18} Adsorbosphere column (Alltech Associates, Deerfield, IL, USA). The solvent system used water (doubly distilled and filtered through a 0.45 μm membrane filter) with 0.05% TFA (solvent A) and 10% acetonitrile in methanol with 0.05% TFA (solvent B). Samples for HPLC analysis were dissolved in HPLC solvent (30% B) and injected on the column using a Rheodyne injector (Cotati, CA, USA) with a 100 μl loop.

Reverse isotope dilution experiments

Four solutions containing a constant concentration of (9,12,12-$^2H_3$)cortisol (0.62 ng/μl) and increasing concentrations of unlabeled cortisol (0, 0.558, 1.178, 3.038 ng/μl) were prepared. One hundred microliters of these solutions were injected on column and analyzed by HPLC–CRIMS. Analyses were performed in triplicate.

Linearity

Five standard solutions containing, 0.20, 0.81, 1.20, 2.45, and 4.81 ng/μl of (9,12,12-$^2H_3$)cortisol were prepared. The labeled cortisol in each solution was diluted to 5% with unlabelled cortisol, which is the target enrichment following the (9,12,12-$^2H_3$)cortisol infusion that was given to the subjects. One hundred microliters of these solutions were injected on column. Analyses were performed in triplicate.

Isolation of steroids from urine

A modification of the method of Shackleton and Whitney [10] was used for isolation of hydrolyzed cortisol metabolites. A 10 ml aliquot of a 0–24 h urine from a patient infused with 30 μg/h (9,12,12-$^2H_3$)cortisol for 31 h was loaded on a C_{18} Maxiclean cartridge (Alltech), washed with 2 ml of water followed by 3 ml of methanol. The methanol fraction was dried and redissolved in 3 ml of acetate buffer and incubated with sulfatase/β-glucuronidase from Helix pomatia (Sigma Type H1, Sigma Chemicals) at 48°C for 48 h. The hydrolyzed urine was loaded on another C_{18} Maxiclean cartridge and washed with 3 ml of 10% methanol in water followed by 3 ml of methanol. The methanol fraction was dried and subjected to HPLC–MS or HPLC–CRIMS analysis. Analyses were performed in triplicate. Although the isotope dilution and linearity work described above was performed on aqueous solutions and this quantitation was done from urine, other experiments, to be published elsewhere, indicated that the recovery of all species determined was greater than 90% for aqueous solutions processed identically to the urine sample. Shackleton and Whitney [10] found near 100% recoveries from urine samples.

RESULTS AND DISCUSSION

HPLC–CRIMS

In hydrogen-scavenged CRIMS experiments

the analyte decomposes to its atoms and the atoms react with the excess hydrogen gas to give products such as CH_4, HCN, and H_2 [3,4]. The hydrogen formed from the analyte is negligible compared to the H_2 reactant gas that is present in great excess. When a deuterium-labeled compound is analyzed, the label is detected as the reaction product HD. At low resolution, the abundance sensitivity for HD^+ at nominal mass 3 is reduced by the signal from H_3^+, an ion formed from ion-molecule reactions of H_2 in the ion source. A resolution of 2000 effectively resolves these two peaks, and the signal at 3.022 represents only the reaction product HD. Such detection is completely selective for HD and highly specific for labeled analytes due to the low natural abundance of deuterium (0.015%).

Preliminary experiments with the Universal Interface in the regular HPLC–MS configuration failed to produce good CRIMS chemistry. The first cause for the problem was the very low pressure in the reaction tube. This low pressure apparently provides an insufficient number of collisions for the particles to dissociate into molecules and for the molecules to be completely decomposed and reformulated into CRIMS products. The pressure in the ceramic tube was increased by two changes. One was removing the second stage of the momentum separator that allowed more helium to enter the reaction chamber. The second was the insertion of a restriction (38 cm of 0.53 mm I.D. fused-silica capillary tubing) between the MS and the ceramic tube to further increase pressure, a configuration identical to how the ceramic tube is coupled to the MS in GC–CRIMS.

Another problem was the increase in background signal due to the transfer of excess solvent vapor through the Universal Interface into the reaction zone. Despite the very high degree of solvent removal by the Universal Interface when operating with its standard settings, the residual solvent still represented a significant background compared to the limit of detection of CRIMS. In these experiments, that limit is about 300 pg/s for our deuterated analytes. One ml/min of solvent represents *ca.* 0.015×10^6 ng/s, and thus we need a greater than 10^6 reduction in solvent to the

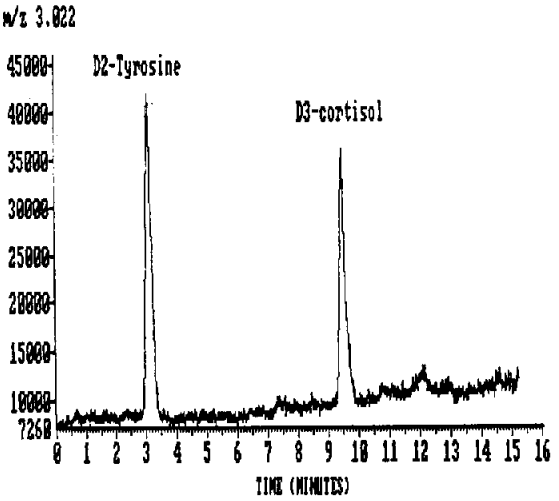

Fig. 2. HD chromatogram in HPLC–CRIMS mode of 100 ng each of (3,5-$^2H_2$)tyrosine, and (9,12,12-$^2H_3$)cortisol. A linear gradient of 40–100% B over 20 min was used for elution.

point where its contribution is negligible compared to a < 1 ng/s analyte flow-rate. The excess solvent transferred was reduced further by increasing the carrier helium flow about 4-fold and by increasing the temperature of the Universal Interface nebulizer from 80°C to 100°C.

A number of experiments to characterize HPLC–CRIMS performance were done. Firstly, the effects of analyte structure and variation in solvent composition on the MS response were investigated. Secondly, the effect of signals from naturally abundant deuterium in unlabeled compounds was examined. Thirdly, the linearity of the MS response to analyte concentrations was measured.

To investigate the effect of analyte structure and solvent composition on CRIMS response, two structurally different compounds, a deuterated amino acid (tyrosine) and a deuterated steroid (cortisol) were analyzed. Fig. 2 shows the HD chromatogram obtained with a 50:50 (w/w) mixture of (3,5-$^2H_2$)tyrosine and (9,12,12-$^2H_3$)cortisol (molar ratio of $^2H = 1.33$). The ratio of the areas of the (3,5-$^2H_2$)tyrosine peak to the (9,12,12-$^2H_3$)cortisol peak was 1.37 (R.S.D. = 1.5%, $n = 6$). Chromatography under isocratic conditions (35% B) gave a peak area ratio of 1.38

TABLE I

RESULTS OF REVERSE ISOTOPE DILUTION MEASUREMENTS

Total F(μg)	% Labeled F	Mean MS response (ADC Units)	S.D.
3.10	2	17349	451
1.24	5	17526	332
0.62	10	17069	444
0.062	100	17389	365

(R.S.D. = 0.9%, n = 4). These values indicate the proportionality of the CRIMS response to the molar deuterium concentration. Since the HD signals were produced by two different compounds, it can be concluded that the analytical system is not significantly affected by the nature of the analyte. This is consistent with many observations with GC–CRIMS [2–4,8,11–13]. In addition, since the composition of the solvent system at the time the two compounds elute is different (40% organic for tyrosine and 60% organic for cortisol) it can be concluded that the system is not significantly affected by the solvent system composition. The detection limits (signal-to-noise ratio = 3) for the two compounds were 5 to 8 ng.

A reverse isotope-dilution experiment was performed to investigate whether unlabeled analyte had any effect on the HD signal. The signals produced by 62 ng of (9,12,12-$^2H_3$)cortisol at different dilution levels (from 100% labeled to 2% labeled) were compared. Table I shows the results of the effect of dilution on the HD signals. These data show that the natural deuterium content of cortisol has no apparent effect on the HD signal at the dilutions studied.

Linearity experiments were performed using five concentrations of (9,12,12-$^2H_3$)cortisol diluted twenty-fold with unlabelled cortisol. A plot of amount injected *versus* mass spectrometric response was linear from 20 ng to 480 ng (9,12,12-$^2H_3$)cortisol. Linear regression analysis gave a line described by, Response = 0.938 × Amount Injected (ng) + 18. The standard errors and S.D.s for the slope and y-intercept were: 0.031 and 0.068; and 2.018 and 4.512, respectively. The correlation coefficient was 0.997. For the measurements at each concentration, the R.S.D. values were less than 3% (n = 3).

HPLC–CRIMS detection of cortisol metabolites in urine

Cortisol is one of the most important endogenous compounds. A number of stable-isotope experiments have been conducted regarding its production and metabolism [14–16]. One of these was conducted by Linder *et al.* who studied plasma cortisol levels of patients infused with a level of (9,12,12-$^2H_3$)cortisol equal to about 5% of the daily cortisol production [15]. We obtained urine from these patients to evaluate the ability of HPLC–CRIMS to survey labeled urinary cortisol metabolites.

CRIMS analysis of cortisol metabolites was done in three steps: development of a separation method that is fast and can separate the metabolites; identification of metabolites from urine by retention times and mass spectra; and optimization of HD detection for the labeled cortisol metabolites.

Fig. 3 shows the HPLC–MS chromatogram

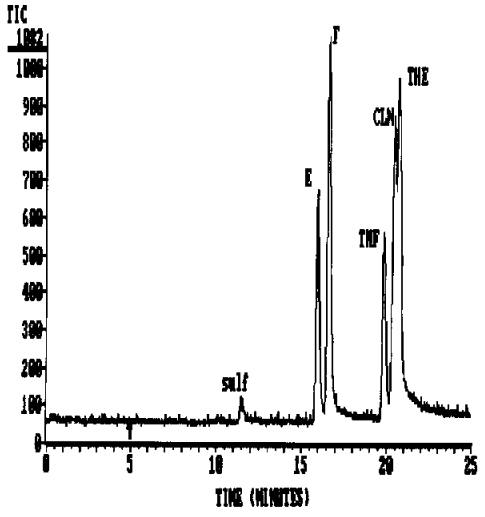

Fig. 3. TIC plot for HPLC–MS mode of 1 μg each of cortisone-21-sulfate (sulf), cortisone (E), cortisol (F), tetrahydrocortisol (THF), cortolone (CLN), and tetrahydrocortisone (THE). A linear gradient of 30–100% B over 35 min was used for elution.

obtained when a solution containing one microgram of each unlabeled metabolite was injected. From this figure it can be seen that the reversed-phase method separates the main classes of cortisol metabolites; THE; THF (THF and 5α-THF are not separated); cortolone (α and β are not separated); cortisone-21-sulfate; cortisol; and cortisone, although slower gradients are required for individual compound quantitation (see Fig. 6). The MS response of the sulfate conjugate is significantly lower than that of the unconjugated metabolites. The much better response of this conjugate observed in the CRIMS mode (not shown) indicates that the poor MS response in the regular HPLC–MS mode is not caused by the poor transport of the conjugates but due to the low ionization of these molecules in the electron impact (EI) ionization mode or their decomposition to masses below our scan range. These observations suggest that HPLC–MS analysis of the unconjugated cortisol metabolites from urine should be feasible.

Fig. 4 shows the total ion chromatogram (TIC) for the HPLC–MS separation of steroids isolated from deconjugated urine of a patient infused with labeled cortisol. A closer inspection of this complex chromatogram reveals the presence of several prominent peaks with retention times corresponding to the retention times of cortisol metabolites. However, only three peaks, labeled A, B, and C, showed spectra similar to cortisol metabolites (Fig. 5). The mass spectra are characterized by very good signal-to-noise ratios and intense molecular ion peaks. The relative intensities of the molecular ion peaks are especially impressive since the EI mass spectra of these compounds, obtained by conventional sample introduction techniques, are dominated by $[M-n(H_2O)]^+$ peaks [17]. Based on the retention times and comparison of their mass spectra with mass spectra of reference compounds, the peaks

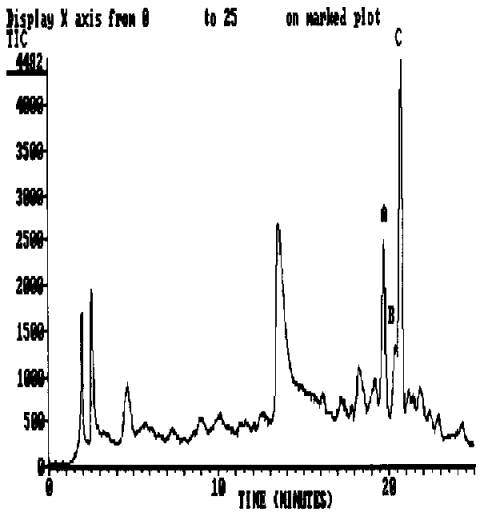

Fig. 4. TIC plot in HPLC–MS mode of a sample isolated from deconjugated urine from a patient during infusion with deuterated cortisol. A linear gradient of 30–100% B over 35 min was used for elution.

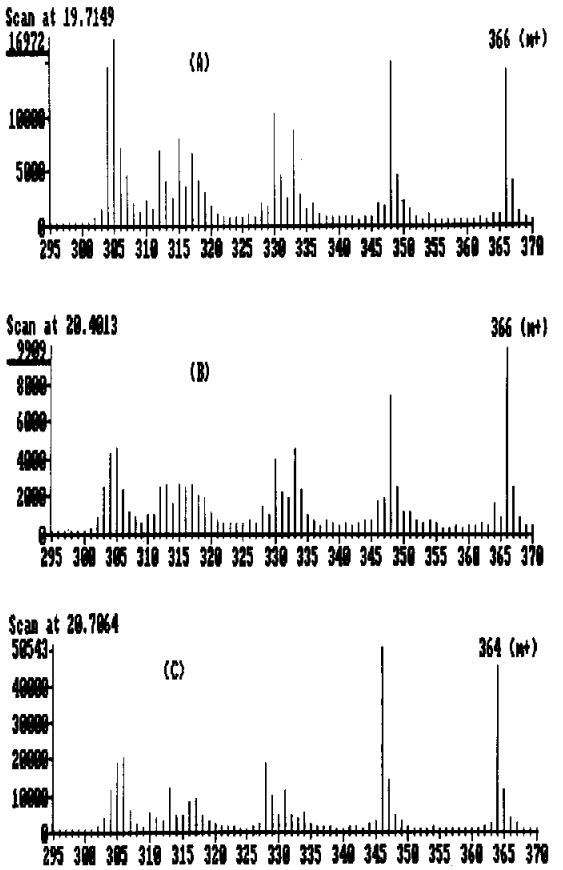

Fig. 5. Mass spectra of peaks A, B, and C in human urine, corresponding to the retention times of tetrahydrocortisol, cortolone, and tetrahydrocortisone, respectively.

were identified as THF, cortolone and THE. From the EI spectra it was difficult to show the existence of the expected 5% labeling in any one of the metabolites identified. This is a disadvantage as the presence of labeling would have been a positive proof that these metabolites are derived from administered cortisol and not from endogenous steroids.

The HPLC–CRIMS analysis of the urine sample previously analyzed by HPLC–MS produced the chromatogram shown in Fig. 6b. Here, a multistep linear gradient was used to achieve better HPLC separation. The retention times of the peaks in the HD chromatogram corresponded with THF, CLN, and THE (Fig. 6a). Neither cortisol nor any other cortisol metabolite, including the minor products α- and β-cortols (not shown) that eluted at retention times different than the ones in the HPLC–CRIMS chromatogram of Fig. 6b, were observed. The presence of deuterium in these peaks proved that they were metabolites of the infused deuterated cortisol.

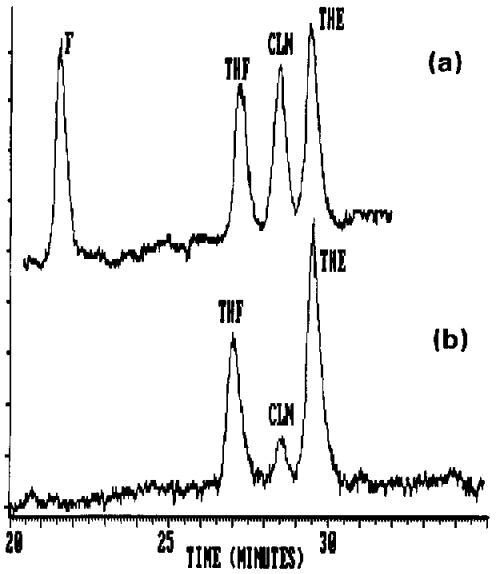

Fig. 6. (a) HPLC–CRIMS chromatogram of cortisol and metabolite standards. The non-specific reaction product C_2H_2 at m/z 26 was used for monitoring total carbon. (b) Deuterium selective chromatogram in HPLC–CRIMS mode of the sample seen in Fig. 4. A multi-level linear gradient of: 30–50% of B over 20 min; 50–70% of B over 40 min, and 70–100% of B over 10 min was used for elution in both (a) and (b).

Based on peak areas, the relative abundance of THF, CLN and THE were 37%, 7.7%, and 55.3%, respectively (R.S.D. $< 1.5\%, n = 3$). The results of the reverse isotope-dilution study are taken as assurance that for these metabolites, where deuterated standards were not available, the observed HD signal is a valid assessment of the labeled materials and is not due to artifacts from the unlabeled analogs.

The use of a metabolic tracer requires that an analytical method be available to recognize the traced species. For nonradioactive tracers following chromatographic separations, the traced species are usually found either by differences in control and treated specimens, or by some type of pattern recognition of isotopic multiplets. This is frequently successful for major metabolites that are present in relatively high concentrations, but it is not in general an approach to comprehensive metabolite determination as is the use of radioisotopes.

These experiments show that the coupling of the Universal Interface and CRIMS provides a method for isotope-selective detection using HPLC separations. The first demonstration of this capability showed that ^{13}C and ^{15}N could be selectively detected following HPLC separations [6]. Here, we demonstrate good analytical qualities for deuterium-selective detection. The detection limit we describe here was obtained with a 20-year old mass spectrometer. We presume that considerably improved performance would result if the HPLC–CRI was attached to a modern instrument. CRIMS has also been used in sulfur- [11,12], chlorine- [8,13], and ^{14}C- [1,4] selective modes with GC as the separating method. The principles of CRIMS chemistry that allowed those GC-based assays should be applicable to the present HPLC–CRIMS instrumentation and therefore provide a substantial range of selective detection strategies following HPLC separations. The enhanced selectivity of CRIMS compared to other types of HPLC detectors should allow comprehensive studies of metabolism in a direct and unambiguous fashion. Finding only the three previously characterized metabolites speaks to the long history and substantial effort directed to

cortisol research. In contrast, with GC–CRIMS in either the ^{15}N- or Cl-selective detection mode, we recently found 14 metabolites of the triazolobenzodiazepine anesthetic drug, midazolam, where the literature has only described four [18].

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